IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of
Yoshiaki Nishiya
Serial No. 10/607,916 Group Art Unit 1641
Filed June 27, 2003 Examiner DO, PENSEE T
For : MAGNETIC CARRIER FOR BIOLOGICAL SUBSTANCE, PRODUCTION METHOD THEREOF AND ISOLATION METHOD OF BIOLOGICAL SUBSTANCE USING SAME

TRANSLATOR'S DECLARATION

Honorable Commissioner of Patents and Trademarks
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Sir:

I, Ritsuko Arimura, declare:

That I am well acquainted with both the Japanese and English languages;

That the attached document represents a true English translation of Japanese Patent Application No. 2002-188140 (filing date June 27, 2002); and

That I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed this 21st day of December, 2007.

Ritsuko Arimura

(Translation)

J A P A N P A T E N T O F F I C E

This is to certify that the annexed is a true copy of the following application as filed with this Office.

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【Title of the Invention】 Magnetic Carrier for Nucleic Acid Binding and Production Method Thereof

【Claims】

【Claim 1】 A magnetic carrier for nucleic acid binding having an average particle size of 0.1-0.5 μm, a coercive force of 2.39-11.94 kA/m (30-150 oersted) and saturation magnetization of 30-80 $A \cdot m^2/kg$ (30-80 emu/g), wherein a spherical or granular ferromagnetic iron oxide particle is coated with silica and the amount of the silica coating is 3-100 wt% relative to the ferromagnetic iron oxide particle.

【Claim 2】 The magnetic carrier of claim 1, wherein the ferromagnetic iron oxide particle is selected from the group consisting of a magnetite particle, a maghemite particle and a manganese zinc ferrite particle.

【Claim 3】 A production method of the magnetic carrier for nucleic acid binding of claim 1 or 2, which comprises adding sodium silicate to an aqueous suspension comprising a spherical or granular ferromagnetic iron oxide particle dispersed therein, adding an acid thereto for neutralization to adhere silica to the ferromagnetic iron oxide particle, wherein the amount of sodium silicate on conversion to SiO_2 is 0.3-2 wt% relative to water, and the amount of the ferromagnetic iron oxide particle is 1-10 wt% relative to water.

【Claim 4】 The production method of claim 3, comprising a heat treatment in an inert gas after adhesion of silica to the ferromagnetic iron oxide particle.

【Detailed Description of the Invention】

【0001】

【Technical Field of the Invention】

The present invention relates to a magnetic carrier for nucleic acid binding comprising a ferromagnetic iron oxide particle coated with silica, which is to be used for extracting

a nucleic acid from a biological substance containing the nucleic acid or purifying the nucleic acid, or purifying a nucleic acid amplification product.

【0002】

【Prior Art】

For isolation of a nucleic acid utilizing a magnetic carrier, a method using a superparamagnetic iron oxide having a coating of polymerizable silane capable of covalent binding with affinity molecules (JP-A-60-1564), a method of using a magnetic carrier wherein the surface of the magnetic particle is coated with a cellulose derivative such as nitrocellulose, which is specifically bounded with a single chain nucleic acid of DNA or RNA (WO86/05815) are known.

【0003】

A method comprising ionically binding a magnetized amine microsphere (magnetic microsphere) of a polycationic support and a sugar phosphate main chain having a negative charge for purification, isolation and hybridization of nucleic acid (Japanese Patent Application under PCT laid-open under kohyo No. hei-1-502319), a method of isolating a pure biological substance using a magnetic responsive particle comprising an internal core polymer particle and a magnetically responsive metal oxide/polymer coating uniformly covering the particle (Japanese Patent Application under PCT laid-open under kohyo No. hei-2-501753), and the like are also known.

【0004】

However, according to these methods, the surface of a magnetic particle carrier and a silane coating or polymer are bound by covalent binding and the like, in which case a functional group is often added to the surface of the magnetized particle. This is advantageous for classification or measurement based on specific adsorption of nucleic acids but inconvenient for a solid carrier that non-specifically

adsorbs many nucleic acids and affords high recovery amount.

【0005】

In general terms, when a surface-coated magnetic particle is used as a solid phase carrier for the isolation of nucleic acid, large particles having a diameter of not less than 20 μm respond even in a weak magnetic field but rapidly produce sedimentation, are poor in operability, and show low binding efficiency with a nucleic acid due to a smaller specific surface area. In contrast, smaller particles having a diameter of not more than 0.1 μm have a larger specific surface area, which in turn improves binding efficiency with nucleic acid, and operability because sedimentation does not occur easily. On the other hand, since smaller particles have lower responsiveness to a magnetic field, a strong magnetic field is necessary to collect the carrier by a magnetic field.

【0006】

From such an aspect, as a solid phase carrier which non-specifically absorbs many nucleic acids and has a high recovery amount, magnetic silica particles which are complexes of superparamagnetic metal oxide with inorganic porous wall substance consisting of silica particles (JP-A-9-19292 and JP-A-2001-78761) and magnetic silica particles having a structure, in which plural fine core particles comprising metal or metal oxide consisting of multiple magnetic domains coated with a film or fine particles of silicon oxide (JP-A-2000-256388), and the like have recently been proposed.

【0007】

【Problems to be Solved by the Invention】

However, since these magnetic silica particles have a structure wherein an agglomerate of a plurality of magnetic particles as core substances is coated with silica, individual magnetic silica particles have larger particle sizes, for example, high values of preferable upper limit of 15 μm to 20

μm. In addition, the number of magnetic core particles contained in individual magnetic silica particles is inconsistent, and the particle size distribution becomes high. As a result, they are inferior in the property of a magnetic carrier for nucleic acid binding, and often show property inconsistency.

【0008】

The present invention aims at solving the above-mentioned problems and obtaining a magnetic carrier for nucleic acid binding which is superior in bindability with nucleic acid and collectability by a magnetic field as well as in the dispersibility upon removal of the magnetic field and elution property of the bound nucleic acid, and capable of improving isolation and purification efficiency of nucleic acid.

【0009】

【Means of Solving the Problems】

The present inventors have conducted an intensive study to achieve the above-mentioned object and found that a magnetic carrier having a good balance between bindability with nucleic acid/collectability by a magnetic field and dispersibility upon removal of magnetic field/elution property of nucleic acid can be obtained by using a ferromagnetic iron oxide particle having a spherical or granular shape and adhering a specific amount of silica near the surface of each ferromagnetic iron oxide particle, and completed the present invention.

【0010】

The ferromagnetic iron oxide particles having a spherical or granular shape are applied to a broad range of use such as those for magnetic recording, toner for copying machines, black color additive to various resins and the like. These ferromagnetic iron oxide particles are requested to uniformly disperse in a medium irrespective of a dry or wet method of use. Therefore, ferromagnetic iron oxide particle undergoes a

surface treatment to improve dispersibility.

For this surface treatment, there are known a method comprising a surface treatment with an inorganic material, and a method comprising surface treatment with an organic material. As a method of surface treatment with an inorganic material, a coating of silica or alumina is generally known.

【0011】

Forming a silica coating near the surface of each ferromagnetic iron oxide particle is not particularly novel. However, for the above-mentioned object, forming of a uniform coating to cover the surface of each particle is important, and the amount is generally several wt% at most relative to a ferromagnetic iron oxide particle. Namely, when improved dispersibility is desired, as the amount of silica to be adhered, several wt% is sufficient. For example, forming of not less than 2 wt% of silica does not result in further improvement in the dispersibility. Rather, the amount of redundant silica without magnetism increases, whereby properties are degraded such as decreasing saturation magnetization amount and blackness.

Therefore, there has been no use for a ferromagnetic iron oxide particle adhered with several wt% or more of silica, which has obliterated the study of adhering such large amount of silica.

【0012】

The present inventors have prepared various magnetic carriers having varying amount of silica adhered to each ferromagnetic iron oxide particle and examined the bindability with nucleic acid/collection by magnetic field, dispersibility upon removal of magnetic field/elution property of nucleic acid, and unexpectedly found that a magnetic carrier coated with a far greater amount of silica than the amount of silica considered to be necessary for coating each ferromagnetic iron

oxide particle, with the purpose of imparting dispersibility, shows superior performance not only in bindability with nucleic acid/collection by magnetic field, but also in dispersibility upon removal of magnetic field/elution property of nucleic acid and completed the present invention.

That is, the present inventors have found that a magnetic carrier having an average particle size of 0.1-0.5 μm obtained by coating a spherical or granular ferromagnetic iron oxide particle with silica in an amount of 3-100 wt%, which is far greater than the amount conventionally considered to be optimal for imparting dispersibility, has dramatically high bindability with nucleic acid.

【0013】

Due to the nature of nucleic acid showing preferential binding with silica, a greater adhesion amount of silica generally means a greater amount of binding with nucleic acid. In conventional magnetic carriers, however, a flock consisting of plural magnetic particles having a smaller particle size before coating with silica is coated with silica. Therefore, the average particle size of the resulting magnetic carrier becomes greater (0.5-15 μm), and the surface area effective for binding with nucleic acid becomes smaller.

In such magnetic carrier, adhesion of a large amount of silica only increases the film thickness of the silica layer on the surface, thus failing to substantially increase the surface area of the silica layer effective for binding with nucleic acid.

【0014】

The present inventors have studied the structure of a most suitable magnetic carrier for nucleic acid binding. As mentioned above, in conventional plural magnetic particles with silica formed thereon, an increased amount of silica formed thereon results in a greater thickness of silica layer on the

surface, and the surface of silica effective for binding with nucleic acid cannot be substantially increased.

In other words, in conventional magnetic carriers, a greater amount of silica contained in the carrier barely affords a nucleic acid extraction efficiency-improving effect. Thus, it was found that the amount of nucleic acid binding can be increased by directly adhering silica to ferromagnetic iron oxide particles and, for this to be achieved, it is effective to make smaller magnetic carrier wherein silica is adhered.

【0015】

As a magnetic carrier wherein this silica is adhered to each ferromagnetic iron oxide particle, a spherical or granular particle having an average particle size of 0.1-0.5 μm is most suitable, particularly preferably 0.12-0.45 μm. When the average particle size is within the above-mentioned range, a magnetic carrier having the best balance which simultaneously achieves bindability with nucleic acid/collectability by magnetic field and dispersibility upon removal of magnetic field/elution property of nucleic acid can be obtained.

In contrast, when the average particle size is smaller than the above-mentioned range, bindability with nucleic acid improves, but collectability by magnetic field and redispersibility upon removal of magnetic field tend to be degraded. Further, when the average particle size exceeds the above-mentioned range, a surface area of the particle becomes small, thus binding efficiency with nucleic acid tends to be lowered.

As long as the average particle size is within the above-mentioned range, particles having a structure wherein plural ferromagnetic iron oxide particles are coated with silica may also be contained.

【0016】

The magnetic carrier bound with a nucleic acid via silica

is collected by a magnet and the like. The collectability depends on the amount of the saturation magnetization of the magnetic carrier, wherein a greater amount of saturation magnetization results in higher improvement of collectability. It was found that, in the magnetic carrier of the present invention, when the amount of silica adhesion is set to the range of 3-100 wt%, the saturation magnetization amount decreases to some extent, but the decrease does not substantially influence the collectability with a magnet.

【0017】

The magnetic carrier collected with a magnet is transferred to a different solution and allows elution of the nucleic acid bound with silica into this solution. The magnetic carrier which has been flocculated when collected with a magnet is required to disperse easily in the solution when separated from the magnet.

While the magnetic carrier of the present invention is a fine particle having an average particle size of 0.1-0.5 μm, the amount of adhesion of silica is as much as 3-100 wt%. The silica effectively acts as steric hindrance, and prevents magnetic flocculation of individual magnetic carriers to show superior dispersibility.

The nucleic acid bound with silica is released from the binding with silica in the solution and is eluted in the solution. The magnetic carrier of the present invention shows fine elution property and the amount of the eluted nucleic acid increases as the binding amount increases, which improves the extraction efficiency of the nucleic acid.

【0018】

Next, as for a coercive force of a magnetic carrier, a greater coercive force generally causes a greater flocculation force between magnetic carriers, which in turn degrades dispersibility of the magnetic carrier during elution of

nucleic acid from the magnetic carrier. As a result, the elution property of the bound nucleic acid from the magnetic carrier decreases and the extraction efficiency of the nucleic acid tends to become lower.

In the magnetic carrier of the present invention, since silica is adhered to each ferromagnetic iron oxide particle, the coercive force of the magnetic carrier is almost determined by the coercive force of the ferromagnetic iron oxide particle. The present inventors have studied the optimal range of coercive force that does not influence the nucleic acid extraction, and found that no practical problem occurs when the coercive force is within the range of 2.39-11.94 kA/m (30-150 oersted)

【0019】

In other words, it was found that when a coercive force of a magnetic carrier exceeds 11.94 kA/m (150 oersted), dispersibility of the magnetic carrier lowers. However, when the coercive force is not more than 11.94 kA/m (150 oersted), no practical problem occurs. While low coercive force of magnetic carrier poses no particular problem, a coercive force lower than 2.39 kA/m (30 oersted) unpreferably requires setting the ferromagnetic iron oxide particle to have a shape and structure not suitable for the object of the present invention, such as a larger particles size and the like.

【0020】

Next, while the saturation magnetization of a magnetic carrier is determined based on the saturation magnetization of ferromagnetic iron oxide particle and the amount of silica, the range of 30 $A \cdot m^2/kg$ (30-80 emu/g) is most suitable. When it is less than 30 $A \cdot m^2/kg$ (30 emu/g), the collectability of a magnet tends to decrease. When it is greater than 80 $A \cdot m^2/kg$ (80 emu/g), the amount of adhered silica decreases, which tens to facilitate agglomeration of magnetic carrier and cause lower

dispersibility.

Thus, a best-balanced magnetic carrier can be obtained, which simultaneously realizes bindability with nucleic acid/collectability by magnetic field and dispersibility during removal of magnetic field/elution property of nucleic acid, when the coercive force is within the range of 2.39-11.94 kA/m (30-150 oersted) and the saturation magnetization is within the range of 30-80 $A \cdot m^2/kg$ (30-80 emu/g).

【0021】

As for the ferromagnetic iron oxide particle, the present inventors have examined the compatibility of various ferromagnetic iron oxide particles as a magnetic carrier for nucleic acid extraction through long years of experience in the development of magnetic material for magnetic recording. As a result, they have found that magnetite particle (Fe_3O_4), maghemite particle (γ-Fe_2O_3), and manganese zinc ferrite particle ($MnZnFe_2O_4$) are most suitable as the ferromagnetic iron oxide particle. It has also been found that, of these ferromagnetic iron oxide particles, iron oxide having a divalent iron ion, such as magnetite particle and the like, may be an intermediate iron oxide of magnetite and maghemite, which is deviated from the above-mentioned stoichiometry to the extent the crystal structure can be maintained.

【0022】

Of the above-mentioned ferromagnetic iron oxide particles, magnetite particles have high saturation magnetization and particularly suitable as the ferromagnetic iron oxide particles for the magnetic carrier of the present invention.

The shape of the ferromagnetic iron oxide particle may be various shapes such as needle, plate, sphere, granule, ellipse, cube and the like, where the shape of particle has an influence on the dispersibility of magnetic carrier when eluting a nucleic acid from a magnetic carrier bound with the

nucleic acid.

【0023】

As mentioned earlier, the amount of silica to be applied is preferably 3-100 wt% relative to ferromagnetic iron oxide particles. When it is less than 3 wt%, the amount of bound nucleic acid and the extraction efficiency decrease unpreferably. When it is higher than 100 wt%, uniform adhesion of silica to the vicinity of individual ferromagnetic iron oxide particles becomes difficult, which in turn reduces the effect provided by an increased amount of bound nucleic acid, and the saturation magnetization amount as a magnetic carrier decreases, thus unpreferably degrading the collectability by a magnetic field.

The adhesion method of silica is to be mentioned below, wherein adhesion of silica to the vicinity of individual ferromagnetic iron oxide particles is important. While conventionally-known methods are effective for coating the whole agglomerate of plural magnetic particles with silica, they are unsuitable for providing the objective magnetic carrier of the present invention.

【0024】

As mentioned above, in the present invention, a well-balanced magnetic carrier can be obtained, which simultaneously realizes bindability with nucleic acid/collectability by magnetic field and dispersibility during removal of magnetic field/elution property of nucleic acid, which is most suitable for extraction or purification of nucleic acid or purification of nucleic acid amplification product, by affording a spherical or granular magnetic carrier having an average particle size of 0.1-0.5 μm wherein silica is coated near the surface of individual ferromagnetic iron oxide particles so that the proportion of the silica relative to the ferromagnetic iron oxide particles will be 3-100 wt%.

【0025】

The ferromagnetic iron oxide particle of the present invention is subject to no particular limitation as to its shape, and may have various shapes, such as sphere, ellipsoid, granular plate, needle, polyhedral and the like. When it is needle or plate, an anisotropic coercive force may emerge. Thus, a spherical, granular or ellipsoidal shape free of such anisotropy is preferable, particularly preferably spherical. That is, the preferable aspect ratio (ratio of maximum length and minimum length as measured in any direction) of the particle is 0.5-2.0. As used herein, by the "spherical" is meant a shape having an aspect ratio of 1.0-1.2 (not less than 1.0 and not more than 1.2), and by the "ellipsoidal" is meant a shape wherein the aspect ratio exceeds 1.2 and is not more than 1.5. By the "granular" is meant one having the same length of particle in all directions, such as a sphere, and one free of particular anisotropy as a whole, though subject to change in length depending on the direction, such as ellipsoid having a greater length in only one direction.

【0026】

【Mode of Embodiment of the Invention】

The magnetic carrier of the present invention is a spherical or granular magnetic carrier having an average particle size of 0.1-0.5 μm wherein silica is coated near the surface of individual ferromagnetic iron oxide particles so that the proportion of the silica relative to the ferromagnetic iron oxide particles will be 3-100 wt%, which is a well-balanced magnetic carrier simultaneously realizing bindability with nucleic acid/collectability by magnetic field and dispersibility during removal of magnetic field/elution property of nucleic acid, and most suitable for extraction or purification of nucleic acid or purification of nucleic acid amplification product.

【0027】

As the ferromagnetic iron oxide particles, one selected from magnetite particle (Fe_3O_4), maghemite particle (γ-Fe_2O_3), magnetite-maghemite intermediate iron oxide particle and manganese zinc ferrite particle ($MnZnFe_2O_4$) can be mentioned. Particularly, magnetite particle is most preferable. In addition, a ferromagnetic iron oxide particle, wherein silica is coated near the surface thereof and heat treated in an inert gas atmosphere is particularly suitable as a magnetic carrier for extraction and purification of nucleic acid, or for purification of nucleic acid amplification product.

【0028】

Taking a case using magnetite particles as the ferromagnetic iron oxide particles as an example, a production method of the magnetic carrier of the present invention is explained in the following. Magnetic carriers using ferromagnetic iron oxide particles other than magnetite particles can also be produced according to the following method.

【0029】

<Synthesis of magnetite particle>

The magnetite particle can be synthesized by the following method comprising oxidation of iron salt in an aqueous solution. First, to an aqueous solution of divalent Fe ion, in which ferrous sulfate ($FeSO_4 \cdot 6H_2O$) has been dissolved, is added dropwise an NaOH aqueous solution to allow precipitation of ferrous hydroxide [$Fe(OH)_2$]. This ferrous hydroxide suspension is adjusted to pH 9-10 and oxidized by blowing air to grow a magnetite particle.

【0030】

When the pH is smaller than the above-mentioned range, precipitation of magnetite becomes slow. When the pH is greater than the above-mentioned range, goethite (α-FeOOH)

tends to grow. The flow rate of the air and retention temperature of the suspension greatly affect the particle size of the magnetite particle. The flow rate of the air is desirably controlled to 100-400 liter/hour and the retention temperature of the suspension is desirably controlled to 50-90°C.

Generally, a higher flow rate of the air accelerates the crystal growth of the magnetite and the particle size becomes smaller. When the flow rate of the air is too small or too large, substance other than magnetite is easily mixed and precipitated during precipitation. A higher retention temperature causes easy crystal growth of the magnetite and the particle size becomes large. When the retention temperature is too low, goethite (α-FeOOH) particle grows easily.

【0031】

According to the method above, a magnetite particle having an average particle size of not less than 0.05 μm and less than 0.5 μm can be synthesized. The average particle size can be determined by measuring the size of 300 particles (when the size varies depending on the direction, the maximum size) on a scanning electron microscope (SEM) image and calculating the average value.

【0032】

<Adhesion of silica>

Using magnetite particles synthesized in this way as ferromagnetic iron oxide particles, a method of adhering silica to the surface thereof is explained.

The synthesized magnetite particle is sufficiently washed with water, and, without drying, suspended in water to give a suspension. In this case, the mixing ratio of magnetite particle and water is adjusted to make the magnetite particle content 1-10 wt% of water. The magnetite particle content relative to water affects uniform adhesion of silica to the

vicinity of the surface of the individual magnetite particles, and silica is most uniformly adhered when the magnetite particle content is within the above-mentioned range.

That is, when the magnetite particle content relative to water is less than 1 wt%, the concentration is too low and silica is easily precipitated in the part other than the surface of the magnetite particle. When the magnetite particle content relative to water exceeds 10 wt%, the concentration becomes too high and the magnetite particle easily flocculates, making uniform adhesion of silica near the surface of each magnetite particle difficult.

【0033】

Then, sodium silicate (water glass) is added to this suspension in a proportion of 3-100 wt% relative to the ferromagnetic iron oxide particle upon conversion to SiO_2. When the amount of sodium silicate to be added is less than 3 wt%, the amount of silica to be adhered to the vicinity of the surface of the magnetite particle becomes insufficient, reducing the amount of nucleic acid to be bound and degrading the extraction efficiency. When it exceeds 100 wt%, uniform adhesion of silica near the surface of each magnetite becomes difficult, which in turn reduces the effect of increased binding amount of nucleic acid. In addition, the collectability by a magnetic field tends to be degraded because the amount of saturation magnetization as a magnetic carrier decreases.

【0034】

The amount of the above-mentioned sodium silicate to be added is preferably adjusted to 0.3-2 wt% relative to water upon conversion to SiO_2. A method of adhering silica to the vicinity of the surface of each ferromagnetic iron oxide particle is described in detail in the Examples. When silica is precipitated by neutralization reaction by adding an acid

such as dilute hydrochloric acid to an aqueous solution of sodium silicate, the liquid viscosity becomes high. When the viscosity becomes too high, uniform adhesion of silica to the vicinity of the surface of each magnetite particle becomes difficult and, when the viscosity is too low, precipitation of silica becomes difficult.

【0035】

As mentioned above, sodium silicate is added in an amount that makes its proportion 3-100 wt% relative to the magnetite particles based on SiO_2, during which the amount of sodium silicate is preferably adjusted to a proportion of 0.3-2 wt% relative to water based on SiO_2, and the amounts of magnetite particles, sodium silicate and water are preferably adjusted so that the amount of magnetite particles relative to water will be 1-10 wt%.

By adjusting in this way, a magnetic carrier wherein silica is adhered to the vicinity of individual magnetite particles, which is most suitable for the extraction and purification of nucleic acid or purification of nucleic acid amplification product can be obtained. The magnetite particle thus synthesized is thoroughly washed with pure water, filtrated and dried in air at a given temperature for a given time (e.g., 60°C for 8 hrs.).

【0036】

<Heat treatment>

The magnetic carrier thus synthesized shows superior performance as a magnetic carrier for extraction and purification of nucleic acid or purification of nucleic acid amplification product. When this magnetic carrier is heat treated in an inert gas, the property is further improved.

The heat treatment is preferably performed in an inert gas such as nitrogen, argon and the like. The heat treatment may be performed in vacuum. While an oxidizing gas such as air

can be used, a high heating temperature causes oxidation of magnetite particle to easily lower the saturation magnetization. Accordingly, use of an inert gas is preferable.

【0037】

The heat treatment temperature is preferably 200-800°C. When it is lower than 200°C, the effect of heat treatment is small. When it exceeds 800°C, magnetite particles are easily flocculated, thereby easily degrading dispersibility during binding and eluting nucleic acid. While the heat treatment time varies depending on the heat treatment temperature, it is generally preferably 1-10 hrs. When the heat treatment time is too short, a sufficient effect of heat treatment cannot be obtained, and when it is too long, the magnetite particles are easily flocculated.

By such heat treatment, silica more firmly binds near the surface of the magnetite particle, thereby improving crystallinity of silica and bindability with nucleic acid.

【0038】

By the method mentioned above, a spherical or granular magnetic carrier having an average particle size of 0.1-0.5 μm, a coercive force and a saturation magnetization of within the ranges of 2.39-11.94 kA/m (30-150 oersted) and 30-80 $A \cdot m^2/kg$ (30-80 emu/g), respectively, wherein 3-100 wt% of silica relative to magnetite particles is formed near the surface of individual magnetite particles, which is most suitable for the extraction and purification of nucleic acid, or purification of nucleic acid amplification product can be obtained.

【0039】

In the present invention, coercive force and saturation magnetization means the values measured using a vibrating sample magnetometer (manufactured by TOEI INDUSTRY CO. LTD.).

The saturation magnetization can be determined from the magnetization amount when 797 kA/m (10 k oersted) of a magnetic

field is applied. The coercive force can be determined from the value of the applied magnetic field at the time point when the magnetization amount becomes nil, which is obtained by applying a magnetic field of 797 kA/m for magnetization, reducing the magnetic field to nil, applying the magnetic field such that the magnetic field gradually increases in the reverse direction.

【0040】

Regarding the magnetic carrier of the present invention, preferable characteristics for the purpose of using the carrier for extraction or purification of nucleic acid, or purification of nucleic acid amplification product are organized once again into the following (1)-(5).

(1) A magnetic carrier wherein silica in an amount of 3-100 wt% of the ferromagnetic iron oxide particle is adhered near the surface of each ferromagnetic iron oxide particle,

(2) this ferromagnetic iron oxide particle is preferably a magnetite particle,

(3) this magnetic carrier has a spherical or granular shape and has an average particle size of 0.1-0.5 μm,

(4) the coercive force and saturation magnetization of the magnetic carrier are 2.39-11.94 kA/m (30-150 oersted) and 30-80 $A \cdot m^2/kg$ (30-80 emu/g), respectively,

(5) in a preferable production method, silica is adhered in an aqueous suspension of this ferromagnetic iron oxide particle by adjusting the amount of sodium silicate on conversion to SiO_2 to 0.3-2 wt% of water, and the amount of ferromagnetic iron oxide particle to 1-10 wt% relative to water,

(6) after adhesion of silica, the carrier is washed with water, dried and preferably subjected to heat treatment in an inert gas.

【0041】

The magnetic carrier of the present invention is mixed

with a material containing nucleic acid or a nucleic acid extract solution, bound with nucleic acid and separated from the solution using a magnetic field, after which the nucleic acid is eluted from the magnetic carrier bound with the nucleic acid, thereby the nucleic acid is isolated and purified.

The step of mixing the magnetic carrier of the present invention and a sample containing nucleic acid or a nucleic acid extract solution can be performed, for example, using a commercially available vortex mixer or gently reversing or shaking the tube for stirring.

【0042】

The step of separating the magnetic carrier bound with a nucleic acid from liquid using a magnetic field is performed using a magnet. As a magnet, for example, a magnet having a magnetic flux density of about 300 gauss can be used. Specifically, a method comprising collecting magnetic carriers bound with nucleic acid to the side wall by placing a magnet near the side wall of a tube containing a sample comprising nucleic acid or a nucleic acid extract solution, and separating the carriers from the solution such as a nucleic acid extract solution and the like can be employed.

Furthermore, a step of eluting a nucleic acid from a magnetic carrier bound with the nucleic acid comprises washing the magnetic carrier bound with the nucleic acid several times with, for example, an ethanol of about 70%, drying the magnetic carrier, then adding sterile water or a solution having a low ion concentration such as TE buffer and the like to cause elution of the nucleic acid bound with the magnetic carrier from the magnetic carrier.

【0043】

【Examples】

The present invention is explained in detail in the following by referring to Examples, which are not to be

construed as limitative.

【0044】

Example 1

<Synthesis of magnetite particle>

Ferrous sulfate ($FeSO_4 \cdot 7H_2O$, 100 g) was dissolved in pure water (1000 cc). Sodium hydroxide (28.8 g) was dissolved in 500 cc of pure water to achieve equimolar with the ferrous sulfate. Then aqueous solution of sodium hydroxide was added dropwise over 1 hr. while stirring to an aqueous ferrous sulfate solution to precipitate ferrous hydroxide. After the completion of the dropwise addition, the suspension containing the precipitated ferrous hydroxide was heated to 85°C with stirring. After the temperature of the suspension reached 85°C, the reaction mixture was oxidized for 8 hrs. while blowing in air at a rate of 200 L/hr with an air pump to give magnetite particles. The magnetite particles were almost spherical and had an average particle size of about 0.28 μm.

The above-mentioned particle size was determined by measuring the size of about 300 particles on a transmission electron microscopic photograph and calculating a number average thereof.

【0045】

<Adhesion of silica>

After thorough washing of a suspension of the above-mentioned magnetite particle with pure water, the weight of the magnetite particle and water was adjusted to 10 g and 200 g, respectively, without drying. The amount of the magnetite in the suspension after water washing was determined by sampling and drying a part thereof. In this suspension was dissolved 3.6 g of sodium silicate.

While the above-mentioned sodium silicate is alkaline in a dissolution state, when adjusted to near neutral by a neutralization step, it precipitates as silica. Therefore, to

this magnetite particle suspension, in which sodium silicate had dissolved, was dropwise added dilute hydrochloric acid with stirring over about 1 hr. and adjusted to near neutral. After the completion of the dropwise addition, the mixture was stirred for 1 hr. By this step, silica was adhered near the surface of the magnetite particle.

【0046】

In this reaction, the amount of sodium silicate and the amount of magnetite particle, relative to water are important, and when the amount of sodium silicate on conversion to SiO_2 is 0.5-2 wt% relative to water, the liquid used to precipitate silica from an aqueous sodium silicate solution by neutralization has an optimal viscosity, whereby silica can be adhered uniformly near the surface of each magnetite particle. When the amount of magnetite particle relative to water is 1-10 wt%, silica can be preferentially adhered near the surface of the magnetite particle.

【0047】

Then, stirring was stopped to allow natural sedimentation. The supernatant was removed, and after water washing, the resultant solid was filtrated and dried at 60°C for 4 hrs. to give a magnetic carrier for nucleic acid binding.

This magnetic carrier was spherical or granular and had an average particle size of 0.32 μm, a coercive force of 4.78 kA/m (60 oersted) and saturation magnetization of 66.8 $A \cdot m^2/kg$ (66.8 emu/g). The amount of the coated silica was 19.4 wt% of magnetite particle on conversion to SiO_2. Fig. 1 shows an SEM image of the magnetic carrier. From this image, it is observed that silica adheres near the surface of the magnetite particle.

【0048】

Example 2

In the same manner as in Example 1 except that the amount of sodium silicate was changed from 3.6 g to 1.8 g in the

silica adhesion step, silica was adhered to magnetite particles to give a magnetic carrier for nucleic acid binding.

This magnetic carrier was spherical or granular, and showed an average particle size of 0.29 μm, coercive force of 4.38 kA/m (55 oersted) and a saturation magnetization of 75.1 $A \cdot m^2/kg$ (75.1 emu/g). The amount of the coated silica was 9.8 wt% relative to the magnetite particle upon conversion to SiO_2. By SEM, adhesion of silica in the vicinity of the surface of each magnetite particle was observed.

【0049】

Example 3

In the same manner as in Example 1 except that, in the silica adhesion step, the weight of the magnetite particle and that of water were changed from 10 g and 200 g to 10 g and 500 g, respectively, and the amount of sodium silicate was changed from 3.6 g to 14.9 g, silica was adhered to magnetite particles to give a magnetic carrier for nucleic acid binding.

This magnetic carrier was spherical or granular, and showed an average particle size of 0.34 (m, coercive force of 5.97 kA/m (75 oersted) and a saturation magnetization of 60.1 A·m2/kg (60.1 emu/g). The amount of the coated silica was 78.9 wt% relative to the magnetite particle upon conversion to SiO2. By SEM, adhesion of silica in the vicinity of the surface of each magnetite particle was observed.

【0050】

Example 4

The magnetic carrier obtained in Example 1 was subjected to a heat treatment in nitrogen gas at 500(C for 2 hours.

This magnetic carrier was spherical or granular, and showed an average particle size of 0.32 μm, a coercive force of 5.18 kA/m (65 oersted) and a saturation magnetization of 68.3 $A \cdot m^2/kg$ (67.3 emu/g). The amount of the coated silica was 19.4 wt% relative to the magnetite particle upon conversion to SiO_2.

By SEM, adhesion of silica in the vicinity of the surface of each magnetite particle was observed.

【0051】

Example 5

In the same manner as in Example 1 except that the temperature of a suspension containing precipitate of ferrous hydroxide was changed from 85°C to 60°C in the synthesis step of magnetite particle, a magnetite particle having an average particle size of 0.13 μm was synthesized.

Using this magnetite particle and in the same manner as in Example 1, silica was adhered to give a magnetic carrier for nucleic acid binding.

This magnetic carrier was spherical or granular, and showed an average particle size of 0.17 μm, a coercive force of 7.57 kA/m (95 oersted) and a saturation magnetization of 63.4 $A \cdot m^2/kg$ (63.4 emu/g). The amount of the coated silica was 19.8 wt% relative to the magnetite particle upon conversion to SiO_2. Fig. 2 shows an SEM image of this magnetic carrier, wherein adhesion of silica in the vicinity of the surface of each magnetite particle can be observed.

【0052】

Comparative Example 1

Commercially available maghemite (γ-Fe_2O_3) particles were used as ferromagnetic iron oxide particles. The maghemite particles have an average particle size of about 0.26 μm, a coercive force of 8.76 kA/m (110 oersted) and a saturation magnetization of 83.5 $A \cdot m^2/kg$ (83.5 emu/g).

Water (25 g) was added to suspend said maghemite particles (5 g). Sodium silicate (28 g) was added to this suspension and dissolved. Sorbitan monolaurate (1.44 g) was dissolved in hexane (96 g), and this solution was mixed with the above-mentioned sodium silicate dissolved maghemite suspension. The mixture was stirred by a homomixer to give an

emulsion dispersion.

Ammonium sulfate (64 g) was dissolved in pure water (288 cc), and while stirring the solution, the above-mentioned emulsion dispersion was added dropwise thereto to give a magnetic carrier comprising a maghemite particle coated with silica, which was washed with water and dried at 60°C.

【0053】

The magnetic carrier for nucleic acid binding thus obtained showed an average particle size of about 5.6 μm, which was far larger than that of 0.1-0.5 μm of the magnetic carrier of the present invention. The coercive force was 7.33 kA/m (92 oersted) and saturation magnetization was 22.1 $A \cdot m^2/kg$ (22.1 emu/g). The amount of the coated silica was 260 wt% relative to maghemite particles upon conversion to SiO_2. In this magnetic carrier, the silica coating includes flock of maghemite particles, unlike the magnetic carrier of the present invention wherein silica adheres near the surface of each magnetite particle, as confirmed from the SEM image.

【0054】

The average particle size, coercive force, saturation magnetization and amount of silica coating on magnetite particles (maghemite particle in Comparative Example 1) on conversion to SiO_2, as major properties of respective magnetic carriers for nucleic acid binding, which were obtained by the above-mentioned Examples 1-5 and Comparative Example 1, are collectively shown in the following Table 1.

【0055】

Table 1

	average particle size (μm)	coercive force (kA/m)	saturation magnetization ($A \cdot m^2/kg$)	amount of silica coating (wt%)
Example 1	0.32	4.78	66.8	19.4
Example 2	0.29	4.38	75.1	9.8
Example 3	0.34	5.97	60.1	78.9
Example 4	0.32	5.18	63.8	19.4
Example 5	0.20	7.57	63.4	19.8
Comp. Example 1	5.6	7.33	22.1	260

【0056】

Then, the respective magnetic carriers for nucleic acid binding, which were obtained in the above-mentioned Examples 1-5 and Comparative Example 1 were subjected to the following extraction tests. Nucleic acids were recovered by extraction from biological samples and recovery performance was examined. The results are as shown in Table 2.

【0057】

(A) Reagent for extraction test

(i) A magnetic carrier for nucleic acid binding was dispersed in sterile water to 0.2 mg/ml to give a dispersion solution.

(ii) As a biological sample for isolation of nucleic acid, bacterial cells obtained by culturing *Escherichia coli* [*Escherichia coli* JM109 (available from Toyobo Co., Ltd., TAKARA SHUZO CO., LTD., Invitrogen Corporation and the like)] in 3 mL TB medium/test tube at 37°C for 20 hrs. were used.

(iii) As a solution for nucleic acid extraction, buffer A which is a buffer containing a chaotropic substance [7M guanidine hydrochloride (Nacalai Tesque, Inc.), and 50 mM Tris-HCl (Sigma), pH 7.5] was used.

(iv) As a washing solution, buffer A which is a buffer containing a chaotropic substance [7M guanidine hydrochloride

(Nacalai Tesque, Inc.), 50 mM Tris-HCl (Sigma), pH 7.5] was used.

(v) As an agent for removing a high concentration salt, a 70% ethanol solution and an acetone solution were used.

(vi) As a solution for elution to recover nucleic acid bound with the magnetic carrier for nucleic acid binding, sterile water was used.

【0058】

(B) Extraction test method

(1) The bacterial cell turbidity (OD660) was measured and the bacterial cells (OD660; 1.0) were prepared by centrifugal separation in an Eppendorf tube for 1.5 cc. Then, a solution for nucleic acid extraction (1,000 μl) was injected and mixed.

(2) Thereafter, a dispersion (20 μl) of the magnetic carrier for nucleic acid binding was added.

(3) While mixing every about 2 min., the mixture was left standing at room temperature for 10 min.

(4) The above-mentioned tube was set on a magnet stand having a shape fitting the 1.5 cc Eppendorf tube to collect the magnetic carrier toward the magnet side.

(5) The solution was sucked with a filter chip and discharged.

(6) The tube was removed from the magnet stand and a washing solution (1 cc) containing guanidine hydrochloride was poured therein.

(7) After thorough mixing with the magnetic carrier, the tube was again placed on the magnet stand, and the solution was discharged in the same manner as above.

(8) The washing step was repeated.

(9) The magnetic carrier bound with nucleic acid was washed with 1 cc of 70% ethanol in the same manner as above and high concentration guanidine hydrochloride was removed.

(10) The residue was again washed with 1 cc of 70% ethanol and 1 cc of acetone.

(11) The above-mentioned tube was set in a heatblock at about 56°C, left standing for about 10 min. and acetone in the tube and in the magnetic carrier was removed by complete evaporation.

(12) Sterile water (100 μl) was added to the magnetic carrier bound with nucleic acid by the above-mentioned method, and the above-mentioned tube was set in a heatblock at about 56°C and the mixture was left standing for 10 min. while mixing every 2 min.

(13) The tube was set on a magnet stand, the solution was recovered by suction with a filter tip, and transferred to a fresh tube. Generally, recovered amount was about 70 μl. Preservation was done at -70°C.

(14) The thus recovered nucleic acid was determined for absorbance (OD 260 nm) with an absorptiometer and nucleic acid concentration was determined. This was multiplied by recovery volume and taken as nucleic acid recovery amount.

【0059】

Table 2

	recovered amount of nucleic acid (ng)
Example 1	2,030
Example 2	1,960
Example 3	1,990
Example 4	2,050
Example 5	2,010
Comparative Example 1	1,070

【0060】

As is clear from the above-mentioned results, the magnetic carriers for nucleic acid binding of Examples 1-5, obtained by adhering silica in an amount of 3-100 wt% of the magnetite particle near the surface of each spherical or granular magnetite particle, had an average particle size of 0.1-0.5 μm, a coercive force and a saturation magnetization of

2.39-11.94 kA/m (30-150 oersted) and 30-80 $A \cdot m^2/kg$ (30-80 emu/g), respectively, were superior in isolation efficiency of nucleic acid, as compared to the magnetic carrier for nucleic acid binding of Comparative Example 1 having a structure where a flock of maghemite particles is included in silica.

【0061】

This is attributable to the fact that, in the magnetic carriers for nucleic acid binding of Examples 1-5, since silica adheres near the surface of each magnetite particle, the amount of silica capable of effectively binding with nucleic acid increases and silica also prevents flocculation of respective magnetite particles, the bindability with nucleic acid/collectability with magnetic carrier by magnetic field and dispersibility of magnetic carrier/elution property of nucleic acid are compatible.

【0062】

【Effect of the Invention】

As mentioned above, the present invention can provide a magnetic carrier having an average particle size of 0.1-0.5 μm, wherein a spherical or granular ferromagnetic iron oxide particle is coated with silica, which shows superior nucleic acid isolating performance capable of simultaneously realizing bindability with nucleic acid/collectability of magnetic carrier and dispersibility of magnetic carrier/elution property of nucleic acid, by setting the coercive force of and saturation magnetization to 2.39-11.94 kA/m (30-150 oersted) and 30-80 $A \cdot m^2/kg$ (30-80 emu/g), respectively.

【Brief Description of the Drawings】

【Fig. 1】 Fig. 1 shows an SEM image (magnification: ×30,000) of the magnetic carrier for nucleic acid binding obtained in Example 1.

【Fig. 2】 Fig. 2 shows an SEM image (magnification: ×30,000) of the magnetic carrier for nucleic acid binding obtained in

Example 5.

【Document】 Abstract

【Summary】

【Problem】 Provision of a magnetic carrier for nucleic acid binding which is superior in bindability with nucleic acid and collectability by a magnetic field as well as in the dispersibility upon removal of the magnetic field and elution property of the bound nucleic acid, and capable of improving isolation and purification efficiency of nucleic acid.

【Solving Means】 A magnetic carrier for nucleic acid binding having an average particle size of 0.1-0.5 μm, a coercive force of 2.39-11.94 kA/m (30-150 oersted) and saturation magnetization of 30-80 $A \cdot m^2/kg$ (30-80 emu/g), wherein a spherical or granular ferromagnetic iron oxide particle is coated with silica and the amount of the silica coating is 3-100 wt% relative to the ferromagnetic iron oxide particle.

【Main Drawing】 none

FIG. 1

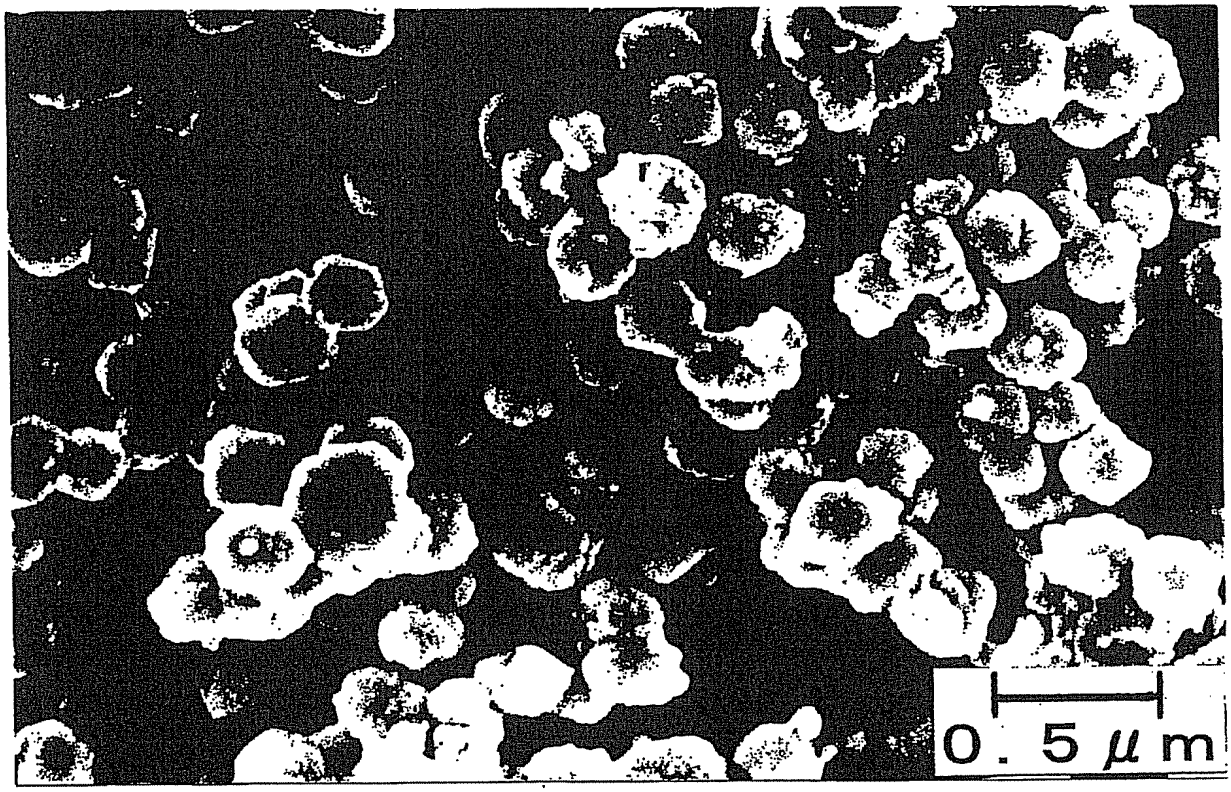

FIG. 2